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Abstract

Arthropods exhibit a large variety of sex-determination systems both at the chromosomal and molecular level. Male heterogamety, female heterogamety, and haplodiploidy occur frequently, but partially different genes are involved. Endosymbionts, such as *Wolbachia*, *Cardinium, Rickettsia* and *Spiroplasma,* can manipulate host reproduction and sex determination. Four major reproductive manipulation types are distinguished: cytoplasmic incompatibility, thelytokous parthenogenesis, male killing and feminization. In this review, we summarize the effects of these manipulation types, and how they interfere with arthropod sex determination in terms of host developmental timing, alteration of sex determination and modification of sexual differentiation pathways. Transitions between different manipulation types occur frequently, which suggests that they are based on similar molecular processes. We discuss how mechanisms of reproductive manipulation and host sex determination can be informative on each other, with a special focus on haplodiploidy. We end with future directions on how study of endosymbiont manipulation of host reproduction can be key to further study of arthropod sex determination.

Key Words: endosymbiont, arthropods, molecular mechanism, sex determination, sexual differentiation, hormonal signaling, epigenetics

Introduction

Arthropods cover over 1,2 million described species that account for about 80% of all known living animal species. They have colonized virtually all habitats on Earth. In line with this broad adaptation to many conditions they exhibit an enormous variety of life histories and reproductive modes. They also show surprisingly large variation and turnover in sex-determination systems. It is therefore a prime group of organisms to study how changes in sex-determination mechanism come about, a current topic in evolutionary biology that is not well understood. A particular aspect of arthropod biology is their frequent infection with microorganisms that can be mutualistic, parasitic or commensal. A specific group are endosymbionts, such as *Wolbachia*, *Cardinium, Rickettsia*, *Spiroplasma* and *Arsenophonus* bacteria, microsporidia and viruses, that manipulate their host's reproduction in a variety of ways [reviewed in Hurst et al., 1996; Werren et al., 2008; Kageyama et al., 2012]. These intracellular parasites are maternally transmitted through the egg cytoplasm. As males are an evolutionary dead end for them, any symbiont having the capability to increase female production is at an advantage and can invade host populations [Partridge and Hurst, 1998; Duron et al., 2008; Werren et al., 2008; Cordaux et al., 2011]. This can be realized through causing thelytokous parthenogenesis, male killing or feminization. As they enhance their own transmission at the expense of their host's fitness, their presence generates genetic conflicts between the two sexes, and possibly an ensuing coevolutionary arms race over offspring sex

[Hurst and Werren, 2001; Werren, 2011]. It has been suggested that such conflict can drive the evolution of changes in host reproduction and sex-determination mechanisms [Werren and Beukeboom, 1998; Stouthamer et al., 2010; Cordaux et al., 2011; Beukeboom, 2012]. Hence, these endosymbionts may be important evolutionary drivers of turnovers in arthropod sex determination.

Here, we review and discuss the current knowledge about manipulative actions of endosymbionts on arthropods. We first briefly summarize the current knowledge about arthropod sex determination and the four major endosymbiont manipulation types of host reproduction. We then move to a specific focus on how symbionts might interfere with host sex determination based on the current knowledge about the molecular bases of host manipulation. We end by proposing future directions on how these reproductive phenotypes may be key to further study of arthropod sex determination. As epigenetic effects are becoming more apparent in insect development, we pay special attention to the possibility of epigenetic regulation.

Arthropod sex determination

Sex determination in arthropods is generally genetically determined by factors on sex chromosomes, with some exceptions in crustaceans in which it is under either temperature or photoperiod control [Bouchon et al., 1998; Cordaux et al., 2011; Kageyama et al., 2012]. Most knowledge comes from insects, where sex determination occurs through a cascade of genes with a highly conserved master switch gene (*doublesex*) at the bottom, but more divergence in the upstream genes (e.g. *transformer*) and the primary signals at the top of the cascade [Wilkins, 1995; Beye et al., 2003; Verhulst et al., 2010; Beukeboom, 2012]. The chromosomal constitutions serve as primary signals and vary between orders. In most insect orders (22 out of 29), the chromosomal constitutions are either XO or XY male heterogamety [Blackman, 1995; Beukeboom and Perrin, 2014]. For instance, most Diptera (flies) and Coleoptera (beetles) have male heterogamety with presence of a Y chromosome (XX/XY), and most Orthoptera

(grasshoppers), Odonata (dragonflies), and Mantodea (mantids) have male heterogamety without a Y (XX/XO). All Lepidoptera (butterflies, moths) and Trichoptera (caddisflies) have female heterogamety (either ZW/ZZ or ZO/ZZ). Hymenoptera (sawflies, wasps, bees and ants) and Thysanoptera (thrips) do not have specific sex chromosomes and reproduce by haplodiploidy (haploid males, diploid females). In addition to these common types of sex determination, more rare variations occur, such as multiple sex chromosomes and X-chromosome or paternal genome loss [Bull, 1985; Sánchez, 2008].

The chromosomal constitutions are translated into different downstream signals that are also diverse among insect orders. In diploids they include X (or Z) chromosome counting elements, dominant masculinizing factors, and dominant feminizing factors. In haplodiploids, allelic complementarity, at one or more sex-determination loci, and maternal effect genetic imprinting have been documented. In most species these signals converge downstream to regulate a key sex-determination gene *transformer*, which directly regulates the sex master switch gene *doublesex* (*dsx*) [Bull, 1985; Nöthiger and Steinmann-Zwicky, 1985; Wilkins, 1995; Marín and Baker, 1998; Raymond *et al*., 1998; Schütt and Nöthiger, 2000; Graham *et al*., 2002; Saccone et al., 2002; Sánchez, 2008; Verhulst et al., 2010; Gempe and Beye, 2011]. Exceptions seem to occur in Lepidoptera where *transformer* has not been found [Suzuki et al., 2001, 2008; see also Geuverink and Beukeboom in this issue]. *Doublesex* in turn regulates genes for sex specific development [Wilkins, 1995; Raymond *et al*., 1998; Schütt and Nöthiger, 2000], and together with the *fruitless* gene regulates sexual differentiation including sexual behaviour [Waterbury et al., 1999; Rideout et al., 2010].

Much less is known about arthropod sex determination outside of the insects, in particular at the level of genes. In crustaceans, heterogametic sex determination appears to be most common [Legrand et al., 1987]. The *transformer* gene has been only identified in the water flea *Daphnia magna*, but does not show sex differences in expression or splicing patterns, rendering it unlikely to be involved in sex determination [Kato et al., 2010]. An important difference from insects is that sex determination in crustaceans is an endocrine process mediated by the androgenic hormone synthetized by the androgenic gland [Ventura et al., 2011]. Basically, individuals have all the genetic information to develop as male or female, but

their fate is determined by a feminizing gene that inhibits the development of the androgenic gland and the synthesis of the androgenic hormone. In absence of the androgenic hormone, female differentiation is induced. In Acari (mites, ticks), both diploidy and haplodiploidy occur, but virtually nothing is known about the genetic regulation of sex determination [Norton et al. 1993; Arakaki et al., 2001]. The same holds for myriapods (millipedes, centipedes) that have male heterogametic sex determination [Fontanetti et al., 2002]. No sex-determination genes have been identified in any of these arthropod groups yet.

Endosymbiont diversity and manipulation types

Over 40% of all arthropods are infected with endosymbionts that live in the cytoplasm of their cells and are vertically transmitted through eggs of females [Werren, 1997; Werren and O'Neill, 1997; Zchori-Fein et al., 2001; Zchori-Fein and Perlman, 2004; Zug and Hammerstein, 2012]. Some of these are obligate mutualists such as *Buchnera* in aphids [Douglas, 1998; Koga et al., 2003], but many others are reproductive parasites. The most prevalent of these host manipulators are the alpha-proteobacteria *Wolbachia pipientis* and *Rickettsia sp*., the bacteroidetes *Cardinium hertigii*, the gamma-proteobacterium *Arsenophonus* and the mollicutes *Spiroplasma poulsonii* and *S. ixodetis*, which belong to very distantly related bacterial clades [Duron et al., 2008]. Four broad categories of host reproduction manipulation are distinguished: induction of cytoplasmic incompatibility between egg and sperm, thelytokous parthenogenetic reproduction, killing of male offspring and feminization of genotypic males [Hurst et al., 2002; Werren et al., 2008; Kraaijeveld et al., 2011]. The molecular genetic details of the mechanisms by which these endosymbionts exert the effects on their hosts are not yet well known. Given the diversity of effects and the variety of microorganisms involved, different questions arise: is this true convergence or are horizontal gene transfers between symbionts involved? If this is convergence among symbionts, is it only at the phenotypic level or also at the mechanistic level? How can we explain the seemingly easy evolution of these manipulations? Do different types of manipulation share common

158 mechanisms? Answering these questions requires a better understanding of the molecular
159 mechanisms at play, which in turn will pave the way to better understand the basic processes
160 of sex determination and their evolution. Before getting into these questions we briefly
161 present the different types of reproductive manipulations. The common theme is that host sex
162 determination is somehow manipulated by the endosymbionts to increase their own
163 transmission, which is by vertical transmission through females. Recent evidence suggests that
164 some of these manipulative actions may be attained by directly interfering with host sex-
165 determination genes [Sugimoto and Ishikawa, 2012; Beukeboom, 2012].
166
167 *Cytoplasmic incompatibility*
168 Cytoplasmic incompatibility (CI) is considered as the most widespread endosymbiont
169 manipulation among arthropods [Werren et al., 2008; Kageyama et al., 2012]. It has been
170 found in Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Isopoda,
171 Trombidiformes, Mesostigmata [Tram and Sullivan, 2002; Werren et al., 2008; Kageyama et al.,
172 2012] (table 1). Despite this broad phylogenetic distribution, CI induction has thus far only
173 been attributed to *Wolbachia* and *Cardinium*. CI is a form of post-zygotic reproductive isolation
174 occurring in crosses between infected males and uninfected females, or when mates harbor
175 different strains of the symbiont [O'Neill et al., 1992; Turelli and Hoffmann, 1995; Werren,
176 1997]. In diploid species, incompatible crosses produce severe cell cycle defects in the male
177 derived pronucleus, resulting in abnormal chromosome condensation at metaphase and
178 aberrant segregation during anaphase of the first mitotic division, which leads to early
179 embryonic mortality [Serbus et al. 2008]. In haplodiploids, CI crosses lead to male-biased
180 offspring sex ratios because elimination of the paternal chromosome set restores haploidy and
181 results in male development [Breeuwer and Werren, 1990, 1993; Breeuwer, 1997;
182 Raychoudhury and Werren, 2012]. However, in some species haploid embryos may also die in
183 an early stage depending on the host species, genotype or the symbiont complement [Vavre et
184 al., 2000, 2001; Perrot-Minnot et al., 2002; Hunter et al., 2003; Mouton et al., 2005], due to
185 the incomplete elimination of paternal chromosomes resulting in aneuploidy, and thus
186 unviable embryos [Tram et al., 2006]. The exact mode of action is not fully understood, but the

current model is based on a chromosome marking effect during male gametogenesis that is rescued in the egg if endosymbionts (inherited from the mother via the egg cytoplasm) of similar type are present [Werren et al., 2008]. CI thus results from a delayed paternal effect as *Wolbachia* or *Cardinium* are not present in the sperm. The sequencing of a CI-inducing *Cardinium* genome was expected to provide insights into the mechanisms of CI, but the recent publication of this genome did not bring more information. Interestingly though, it suggests that CI has an evolutionary independent origin in *Wolbachia* and *Cardinium*, since no recent horizontal gene transfer between these two symbionts has been detected [Penz et al., 2012]. CI-*Wolbachia* can readily spread in populations, because infected females have an advantage over uninfected females in that they are compatible with uninfected and infected males (Werren 1997).

Thelytokous parthenogenesis

Several types of endosymbionts have been found to induce thelytokous parthenogenesis including *Wolbachia*, *Cardinium and Rickettsia* [Werren, 1997, 2008; Giorgini et al., 2010] (table 1). Parthenogenesis induction (PI) by microbes entails making the host reproduction independent of fertilization. This results in progeny that consist entirely of females if the parthenogenesis-induction is 100% effective. Parthenogenetic development of eggs requires special adaptations to the mode of oogenesis, i.e. the diploid complement needs to be restored after meiosis. There are many ways in which this could be accomplished [Suomalainen et al., 1987; Stenberg and Saura, 2009], including several modifications of meiosis, but the mechanisms used by endosymbionts appear rather limited (see below). Moreover, the taxonomic distribution of endosymbiont-induced-thelytokous-parthenogenesis in arthropods is quite restricted. It has thus far only been documented for haplodiploids, like hymenopterans, thrips and mites (table 1). In these groups the endosymbionts cause doubling of the chromosomes in the egg without subsequent cell division. Because of haplodiploid sex determination, the haploid eggs that would normally develop into males are converted into diploid eggs that develop into females [Werren et al., 2008]. In other words the sex reversal is opposite to that of CI: genetic males are converted into genetic females by changing the

chromosome complement of the zygote from haploidy to diploidy. Curing of hosts from their endosymbionts with antibiotics typically results in the production of haploid eggs that develop into males.

Cytological studies on a number of hymenopterans have revealed several different post-meiotic mechanisms of diploidy restoration. In *Trichogramma pretiosum, T. deion*, and *T. nr. deion*, diploidization is due to a segregation failure of the two sets of chromosomes in the first mitotic anaphase [Stouthamer and Kazmer, 1994]. A similar mechanism occurs in *Leptopilina clavipes* [Pannebakker et al., 2004]. In *Muscidifurax uniraptor*, however, the normal first mitotic anaphase is followed by fusion of the adjacent first mitotic nuclei [Gottlieb et al., 2002], a process known as gamete duplication. The result is two identical sets of chromosomes and completely homozygous progeny. In the haploid mite *Bryobia praetiosa*, reproduction is functionally apomictic with all progeny identical in genotype to their mother and heterozygosity being maintained [Weeks and Breeuwer, 2001]. The similar functionally apomictic cloning mechanism was also found in the heterozygous offspring of *Rickettsia*-infected parasitoid wasp *Neochrysocharis formosa* [Adachi-Hagimori et al., 2008].

PI is the ultimate strategy for a maternally-transmitted symbiont: as fertilization is superfluous, fixation of the symbiont within populations or entire species is possible. Curing of hosts from their endosymbionts with antibiotics typically results in male production [e.g. Zchori-Fein et al., 2001; Kremer et al., 2009]. However, restoration of sexual lines has yet proved impossible in species in which the endosymbiont is fixed. Sexual traits have decayed either both in males and females, or males partially retain functionality. Two alternative explanations have been proposed. The neutral mutation hypothesis states that if traits involved in sexual reproduction are neutral under asexuality, relaxed selection might take place and allow mutations to accumulate, for instance, in male sexual traits such as courtship behavior and fertility. The selective hypothesis considers that sexual traits decay can be selected for in females. First, if sexual traits are costly and no longer provide fitness benefits, they are expected to be strongly negatively selected. This applies stronger to female than male sexual traits, like pheromone production, spermatheca functionality, and egg fertilization, because the males are absent under asexuality [Fong et al. 1995; Schwander et al. 2013]. Second, when *Wolbachia* infection

remains polymorphic through inefficient transmission of the symbiont, nucleo-cytoplasmic conflict over sex-ratio may select nuclear alleles for higher male production, referred to as "virginity mutants", which can be achieved through losing the ability to use sperm, or losing the ability to mate [Stouthamer et al., 2010]. Whatever the mechanism at play, PI-symbionts are associated with loss of traits involved in the normal process of sexual reproduction, and this process can be either neutral or actively selected for, which opens up the possibility that endosymbionts take over the role of genes involved in sex determination and sexual differentiation.

Male killing

Male killing (MK) is induced by a large diversity of endosymbiont taxa and found in a variety of arthropod host orders (table 1). *Wolbachia, Spiroplasma, Rickettsia*, *Arsenophonus*, *Flavobacteria*, as well as microsporidia have all been reported to cause male killing [reviewed in Hurst and Jiggins, 2000; Kageyama et al., 2012]. Male-killing occurs if sons of infected mothers are killed by the endosymbiont during development [Bonte et al., 2008; Werren et al., 2008]. Endosymbiont-induced male lethality has been reported from six different arthropod orders, i.e. Coleoptera, Diptera, Pseudoscorpiones, Hemiptera, Lepidoptera and Hymenoptera [Werren et al., 2008; Kageyama et al., 2012] (table 1). The MK phenotype is variable and can be divided into two broad categories according to the timing of action: early male killing at embryonic stages and late male killing at late larval or early pupal stages [Hurst, 1991; Kageyama et al., 2007]. Of interest, male-killing is found in species with either male or female heterogamety, as well as haplodiploidy, which suggests, together with developmental timing variation, that male-killing is the outcome of different molecular mechanisms (table 1; fig. 1). Early male-killing is typically encountered in species where intra-brood competition is high; killing brothers allows sisters to have more resources for survival. Late male-killing is associated with parasites having both vertical and horizontal transmission. The microorganisms gain the maximal benefit from it, because male hosts which do not contribute to vertical transmission are killed at the late larval stage when the number of infected cells is maximal allowing for the maximal horizontal transmission [Hurst, 1991; Kageyama et al., 2007;

Nakanishi et al., 2008]. Importantly, the presence of male-killing selfish elements leads to selection for host resistance. This is notably what occurred in the butterfly *Hypolimnas bolina* where Asian populations harbor a dominant resistant allele to the male-killing phenotype, although the mechanistic details are not yet known [Hornett et al., 2008]. Interestingly, the rapid spread of resistance has been monitored in natural populations of the South Pacific, highlighting both the dynamic nature of these interactions and the intensity of the selective pressures generated by reproductive manipulators [Charlat et al., 2007].

Feminization

Conversion of genotypic males into phenotypic and functional females is known as feminization (FM) [Bouchon et al., 1998; Kageyama et al., 1998]. Endosymbiont-induced feminization has been reported from seven arthropod orders: Lepidoptera, Hemiptera, Hymenoptera, Thrombidiformes, Isopoda, Ephemeroptera and Amphipoda [reviewed in Werren et al., 2008; Kageyama et al., 2012]. Feminization is associated with different sex-determination mechanisms in these groups, such as male or female heterogamety, haplodiploidy, and some unknown mechanisms for crustacean species (table 1). Feminization seems to be more frequent in crustaceans than in insects, which could be due to the easiness to manipulate sexual phenotypes in the former. Indeed, simple manipulation of hormonal levels in crustaceans leads to sex reversion. In the well-studied woodlouse *Armadillidium vulgare* (Isopoda), *Wolbachia* feminizes ZZ males by interfering with the production/perception of the androgenic hormone from the male developmental gland during sexual differentiation [Bouchon et al., 2008; Cordaux et al., 2011]. This resembles the shrimp *Gammarus duebeni*, in which microsporidian parasites, such as *Octosporea effeminans* and *Nosema granulosis*, change males into functional females [Bulnheim and Vávra, 1968; Terry et al., 1998; Rodgers-Gray et al., 2004]. Feminization has also been found in insects where different mechanisms could be at play, such as disrupting methylation patterns and genetic imprinting in the male-heterogametic leafhopper *Zyginidia pullula* [Negri et al., 2006, 2009], or altering splicing of *doublesex* in the female-heterogametic butterfly *Eurema mandarina* [Narita et al., 2007]. Feminization also occurs in haplodiploid species. Giorgini et al. [2009] found that

303 in *Encarsia hispida*, curing from *Cardinium* does not lead to haploid but diploid males,
304 suggesting that the endosymbionts are not responsible for genome duplication
305 (parthenogenesis) but rather cause feminization of diploid males. Moreover, in the *Cardinium*
306 infected mite *Brevipalpis phoenicis*, consisting exclusively of haploid females, Weeks et al.
307 [2001] reported that curing of the bacterium changes haploid daughters into haploid sons.
308 Under endosymbiont-induced feminization, scarcity of males within host populations
309 generates a strong nucleo-cytoplasmic conflict. Resistance forms have been detected in some
310 cases, notably in *A. vulgare*. In this species, together with masculinizing genes, other
311 feminizing factors have been evidenced, but encoded by the nuclear genome [Juchault and
312 Mocquard, 1993]. There is some evidence that this nuclear feminizing factor originates from a
313 horizontal gene transfer from *Wolbachia*. The *A. vulgare* system is a good illustration of the
314 dynamic nature of sex determination where female and male heterogamety are evolving in
315 response to feminizing *Wolbachia* [Cordaux et al., 2011]. The high diversity and dynamics of
316 sex determination systems and the absence of sex chromosome differentiation in crustaceans
317 makes it likely that this pattern occurs more widespread in crustaceans [Rigaud et al., 1997].
318
319
320 **Mechanisms of reproductive and sex determination manipulations**

321 With respect to genetic mechanisms we delineate a typology of reproductive manipulations.
322 CI, PI, MK and FM differ in their actions in relation to the timing at which they interfere with
323 the host sex determination and differentiation processes. Taking the master sex switch gene
324 *doublesex* as the central point ("bottleneck in an hourglass"), manipulations can target earlier
325 events constituting the primary signals, *doublesex* itself, or downstream processes including
326 sexual differentiation (fig. 1). This typology integrates phylogenetic and empirical information,
327 and allows us to consider different reproductive-manipulation mechanisms in a phylogenetic
328 context. It indicates that endosymbionts have the potential to undergo rapid evolutionary
329 shifts in phenotypes [Werren, 1997; Jaenike, 2007; Kraaijeveld et al., 2011]. Below we discuss

how mechanisms of reproductive manipulation may be informative for the molecular bases of host sex determination.

Interference with primary signals

Interference with primary sex determination signals concerns notably manipulation of chromosomal behavior. This is clearly established for CI where paternal effects lead to ploidy changes in the early fertilized egg. CI-endosymbionts in diploid arthropod species obviously do not interfere with host sex determination because they cause lethality through haploidization of eggs [Serbus et al., 2008]. However, in haplodiploids, conversion of diploid female eggs into haploid male eggs occurs by changing the zygotic chromosomal constitution that act as primary signal for sex determination. This is very similar to PI-endosymbionts that also act early during sex determination as they alter the number of chromosomal complements at the end of the first or beginning of the second mitotic division. As transcription is probably limited at that time, PI is certainly a parental effect, but contrary to CI, it is limited to a maternal effect. It is still unknown how endosymbionts precisely alter the molecular regulation of mitosis to induce diploidization of the host eggs. Why parthenogenesis inducing microbes have not been found in diploid species remains another mystery. One explanation is that PI evolves more easily in haplodiploids because of the pre-existing cellular machinery of full development from unfertilized haploid eggs. The interaction between mechanisms of sex determination and PI endosymbionts are particularly complex and further elaborated in Box 1. The early MK type can also act on the zygotic chromosome constitution that serves as the primary signal in the host sex-determination pathway. In the wasp *Nasonia vitripennis*, *Arsenophonus nasoniae* kills male offspring by blocking maternal centrosome formation during oogenesis [Ferree et al., 2008]. In *Drosophila bifasciata*, infected male embryos show severe defects of chromatin remodeling and spindle organization, a phenotype strikingly similar to the phenotype observed in CI [Riparbelli et al., 2012].

Early acting endosymbionts that alter the chromosomal constitution, a feature of PI, CI and early MK, suggests similar target host genes that have a relatively broad function. This would explain why the manipulations occur in such a diversity of host taxa regardless of their sex-

359 determination system. There are many molecules that endosymbionts could target to change
360 the chromosome constitution of the egg. Of particular interest, CI is associated with impaired
361 histone deposition in the male pronucleus, which could lead to activation of cell cycle
362 checkpoints [Landmann et al., 2009]. Other examples include the inhibition of the proper
363 digestion of cohesions that would result in failure of chromosome separation during meiosis
364 or mitosis [Ferree et al., 2008; Schurko et al., 2009]. A similar effect might be achieved by
365 interfering with the signals that regulate the M checkpoint in the cell cycle. An interesting class
366 of potential target genes are meiosis related genes which code for Argonaute proteins or
367 mitotic division related genes coding for cell cycle proteins [Schurko et al., 2009; Kraaijeveld
368 and Bast, 2012]. Informatively, *Wolbachia*-induced CI can transit to MK (fig. 2), as was found in
369 two *Drosophila* species and two moth species. MK occurred when uninfected males of
370 *Drosophila subquinaria* mated with hybrid females from the cross between *Drosophila recens*
371 females with the CI phenotype and endosymbiont uninfected *D. subquinaria* males [Sasaki et
372 al., 2002, 2005; Jaenike, 2007]. Interestingly, the same transition but in opposite direction,
373 from MK to CI, occurred in the butterfly *Hypolimnas bolina* [Hornett et al., 2008]. The
374 suppression of the MK phenotype in infected individuals resulted in male production, which
375 upon mating with uninfected females induced CI (fig 2). These studies suggest that it is
376 relatively easy to shift between male killing and cytoplasmic incompatibility, and point towards
377 similar mechanisms. Transitions can also occur from PI to CI. In *Asobara japonica*, male
378 offspring produced by PI-*Wolbachia*-infected females induced (moderate) CI against
379 uninfected females [Kraaijeveld et al., 2011] (fig. 2).
380
381 *Direct interference with doublesex*
382 Late acting endosymbionts are associated with sexual differentiation, and must recognize
383 maleness resulting from male specifically expressed genes during development. It is now
384 evident that endosymbionts can directly interfere with the expression of sex determination
385 genes. For example, male killing in the moth *Ostrinia scapulalis,* is accomplished by altering
386 the splicing of *doublesex* [Sugimoto and Ishikawa, 2012]. Altered splicing is also found in the
387 butterfly *Eurema mandarina,* in which *Wolbachia*-infected genetic males (ZZ) are

388 morphologically and behaviorally fully female and completely fertile. The splicing pattern of
389 the sex-determining gene *dsx* changes according to the *Wolbachia* infection status. Intersex
390 individuals express both female and male *dsx* splice variants. The lethal effects normally occur
391 during late embryonic or early larval developmental stages, and might be due to disruption of
392 dosage compensation [Kageyama and Traut, 2004; Narita et al., 2007; Sakamoto et al., 2007;
393 Sugimoto et al., 2010; Sugimoto and Ishikawa, 2012]. It is still unknown whether *Wolbachia*
394 directly acts on *dsx* splicing, or (more probably) on an upstream splicing regulator of *dsx* in this
395 female heterogametic system [Beukeboom, 2012]. In the *Spiroplasma* infected ladybird beetle
396 *Anisosticta novemdecimpunctata*, males are killed in the early embryonic stage [Tinsley and
397 Majerus, 2006], but the genetic mechanism is still unknown, as is true for all MK types in
398 ladybirds [Balayeva et al., 1995; Hurst et al., 1996]. These examples of early MK show that the
399 microbes have evolved different ways of killing males. The MK in *Ostrinia* is the first well
400 documented case of direct seizure of endosymbionts upon host sex-determination genes. Due
401 to being the central gear of the key sex-determination gene*, transformer* is expected to be a
402 particularly likely target for such manipulation in holometabolous insect sex determination
403 [Beukeboom, 2012; Negri and Pellecchia, 2012].
404
405 *Interference during sexual differentiation*
406 Male killing can also occur in the sexual differentiation phase of embryonic or larval
407 development. A functional dosage-compensation complex, a major component of sexual
408 differentiation in *Drosophila melanogaster*, is required for male-killing by *Spiroplasma*.
409 *Spiroplasma* failed to kill males lacking any of the five protein required for proper dosage-
410 compensation [Veneti et al., 2005]. Dosage compensation is tightly connected with sex
411 determination in *Drosophila*, as the gene *sex lethal* which has both a function in dosage
412 compensation and in sex determination as a splice-regulator of *transformer* [Cline, 1984].
413 Although yet speculative, it may be that the MK *Spiroplasma* targets the *sex lethal* gene [Starr
414 and Cline, 2002]. In the mosquito *Aedes stimulans*, *Amblyospora* microsporidia kill males in
415 the fourth larval stage [Andreadis, 1985], which is another example of late male killing.
416 Furthermore, an unknown RNA virus was found responsible for late male killing in the oriental

417 tea tortrix, *Homona magnanima*, in which male death occurs in the larval or pupal stage
418 [Nakanishi et al. 2008].

419 Hormonal signaling pathways are frequently involved in the regulation of symbiotic
420 interactions. In parasitic interactions such as host-parasitoid relationships, they play a central
421 role in synchronizing host and parasite cycles, and manipulation of hormonal signaling by each
422 party has been found [Sagi and Khalaila, 2001; Negri, 2011; Jahnke et al., 2013]. Hormonal
423 signaling as part of sexual differentiation can also be manipulated by endosymbionts. This is
424 apparent in crustaceans where the establishment of the sexes is a hormonal process. Notably,
425 injection of *Wolbachia* in young males of *A. vulgare* induces the hypertrophy of the androgenic
426 gland and the feminization of tissues [Rigaud and Juchault, 1995]. This result indicates that
427 *Wolbachia* may interfere with the androgenic hormone receptors and either antagonize the
428 fixation of the androgenic hormone on these receptors, or decrease their production. The
429 androgenic hormone is related to insulin and/or insulin-like growth factors, which is
430 interesting for two reasons. First, *Wolbachia* has been shown to interact with the insulin
431 pathway in *Drosophila* [Ikeya et al., 2009]. Even though this pathway is not directly involved in
432 sex determination, insulin-like peptides regulate ecdysteroid synthesis, and recent results
433 indicate that 20hydroxyecdysone could play the role of a sex hormone in insects (Negri et al.,
434 2010; Negri and Pellecchia, 2012). Hormonal manipulation seems mostly associated with
435 feminization, but male-killing may also make use of hormonal signals that are different
436 between the sexes. It should however be noted that sex determination in insects is generally
437 considered as a cellular genetic process, and that the importance of hormonal signaling is still
438 under debate [Steinmann-Zwicky et al., 1989; Schütt and Nöthiger, 2000; Negri and Pellecchia,
439 2012]. This is informative for the transition between MK and FM. The phenotype transition
440 from MK to FM is observed in the moth *Ostrinia scapulalis*. Antibiotic treatment induced
441 intersex individuals suggesting that MK-inducing *Wolbachia* were also responsible for
442 feminization [Kageyama and Traut, 2004; Sakamoto et al., 2008; Sugimoto and Ishikawa, 2012].
443 In addition, transition from PI to FM occurred in the parasitoid wasp *Trichogramma kaykai*. In *T.*
444 *kaykai* with PI phenotype, diploid intersex individuals were produced under high temperature,
445 suggesting that PI *Wolbachia* are also responsible for feminization that is *Wolbachia* density

dependent [Tulgetske and Stouthamer, 2012] (fig. 2). A small proportion of diploid males is also regularly detected in the parasitoid wasp *Asobara japonica*, which suggests that PI-*Wolbachia* are required for feminization and that this effect is dependent on *Wolbachia* density [W.J. Ma, unpublished data].

Box 1 Interaction between PI and FM endosymbionts, and haplodiploid host sex determination

The interaction between endosymbionts and haplodiploid host sex determination is complex, because the mechanisms by which diploidization of the egg takes place also affects the outcome. In some cases it dictates whether particular endosymbionts can establish (fig. 3). Several hymenopteran groups have complementary sex determination (CSD) in which sex is determined by the allelic composition of the sex locus: heterozygotes develop into females, hemizygotes and homozygotes into males [Whiting, 1933; Cook, 1993a; Beye et al., 2003]. CSD and PI-inducing endosymbionts that cause gamete duplication are incompatible [Cook, 1993b; van Wilgenburg et al., 2006], because this form of diploidization results in complete homozygosity in most documented species so far [Stouthamer and Kazmer, 1994; Pannebakker et al., 2004; Gottlieb et al., 2002]. The reason is that under CSD diploid homozygotes develop into males whereas female development is required for PI endosymbionts to invade a host. There is indeed a phylogenetic association between absence of CSD and presence of PI endosymbionts [Heimpel and de Boer, 2008]. Interestingly, some CSD species do reproduce parthenogenetically, such as *Venturia canescens*, but in such species the diploidization mechanism is different (e.g. central or terminal fusion) and apparently retains sex locus heterozygosity [Suomalainen et al., 1987; Beukeboom and Pijnacker, 2000; Mateo-Leach et al., 2009]. Functionally apomictic cloning mechanism is also the case for the haploid mite *Bryobia praetiosa* and the parasitoid wasp *Neochrysocharis formosa* [Weeks and Breeuwer, 2001; Adachi-Hagimori et al., 2008].

The other known genetic mechanism of sex determination in Hymenoptera is maternal effect genomic imprinting sex determination (MEGISD). Under MEGISD female development

requires a paternal genome for activation of the *transformer* gene in the zygote, which is silenced on the maternal complement [Verhulst et al., 2010, see also Verhulst and van de Zande in this issue]. It has thus far only been documented for *Nasonia vitripennis* (Chalcidoidea). The broader phylogenetic distribution of the MEGISD model has been challenged, because it is difficult to reconcile with parthenogenetic female reproduction in which a non-imprinted male genome is missing in the egg. One solution would be that the maternally provided imprint is not copied onto the duplicated genome copy during the diploidization process, providing an active *transformer* copy to the zygote without fertilization. Under this assumption, PI endosymbionts would be able to infect species with MEGISD. On the other hand, if the maternal imprint would be passed on, zygotic diploidy would result in males, and PI endosymbionts cannot establish (fig. 3). For other forms of diploidization to establish, such as central and terminal fusion, it is necessary to assume that the endosymbionts can remove the maternal imprint, because fusion of two meiotic nuclei, each with a maternal imprint, would lead to diploid males. Only gamete duplication without imprint copying alleviates the requirement of endosymbiont interference with MEGISD (fig. 3). Further information is needed on the phylogenetic distribution of the MEGISD system before these issues can be solved.

In the chalcidoid *Encarsia hispida*, diploid males are produced when females are cured from *Cardinium*. The type of endosymbiont action, following the above rational, is thus informative for the sex determination mechanism of this species: it may have MEGISD without the maternal imprint copy (fig. 3, see the green lines originating from MEGISD). Taking the opposite argumentation, having MEGISD may have prevented it from being infected by PI endosymbionts (fig. 3, see the red lines originating from MEGISD). How egg diploidization and feminization occurs in this system is not yet known. Removal of the bacteria yields diploid males indicates that egg diploidy is controlled by the host genotype. Assuming MEGISD, one possibility is that *Cardinium* prevents transmission of the maternal imprint to the duplicated genome copy, turning diploid male eggs into diploid female eggs.

Conclusion and future directions

Although there have been several reviews on endosymbiont manipulation of arthropod host reproduction, we have taken a specific focus on the mechanisms by which endosymbionts may interfere with host sex determination. From considering the four major endosymbiont manipulation types, it is clear that the diverse endosymbionts can target the host at different developmental stages, ranging from spermatogenesis stage or the first mitotic division to the late pupal stage. The evolution of similar manipulation types in distantly related endosymbiont taxa shows that convergent evolution has probably occurred repeatedly. Many of the intricacies of endosymbiont-host interactions remain to be discovered because in most instances it is still unknown what developmental pathways are exploited by the endosymbionts to exert their effects on host reproduction. We have proposed that the transitions between endosymbiont phenotypes suggest partly similar mechanisms for apparent divergent phenotypes. We have also argued that the mechanism of endosymbiont manipulation must be considered in the context of the host sex determination mechanism and that both of these processes may be mutually informative on each other.

With the development of Next-Generation DNA sequencing techniques, it is getting easier to acquire genomic information on non-model organisms, which makes the unraveling of the genetic basis of endosymbiont manipulation very promising and exciting. A first question to answer is whether the diversity of effects and the variation of microorganisms involved, reflects true convergence or merely horizontal gene transfer between symbionts. Future studies should compare different endosymbiont genomes for gene composition, as well as compare gene products that might affect developmental pathways of their host [e.g. Moreno et al. 2011]. For instance, the comparison of genomes between *Wolbachia* and *Cardinium* suggests that CI has an evolutionary independent origin in these two symbionts and reveals no evidence for recent horizontal gene transfer [Penz et al., 2012]. Comparison of transcriptomes and proteomes of infected and uninfected hosts may also be rewarding [e.g. McNulty et al. 2012]. Moreover, the integration of knowledge about evolutionary dynamics and genomic

data should make it possible to identify genomic signatures that can lead to the identification of genes involved in host reproduction and sex determination manipulation. Attention should be paid to host sex determination genes such as *transformer* and *doublesex*, candidate targets for disruption by endosymbionts, whose regulation may be altered in several ways, including their sex-specific splicing or imprinting. In addition, cell cycle genes involved in meiosis and mitosis, particularly those related to histone regulation or genes coding for Argonaute proteins are good candidates (Kraaijeveld and Bast, 2012).

There is growing evidence for epigenetic control of developmental processes in insects [Lyko & Maleszka 2011], as well as in host-parasite interactions [Gómez-Díaz et al., 2012]. Given the multiple evolution of reproductive manipulations, it is tempting to propose that these phenotypes may actually be mechanistically very close to other physiological mechanisms involved in host-parasite interactions that could represent pre-adaptations to reproductive manipulations [Vavre et al., 2003]. As reproductive manipulations often involve parental effects, epigenetic manipulation by endosymbionts clearly requires attention. An obvious candidate is chromatin remodeling which can lead to alteration of chromosomal behavior, as well as to variation in gene expression or splicing processes. It is thus possible that many of the symbiont phenotypes rely on epigenetic mechanisms, particularly those related to histone regulation. Moreover, paternal effects of CI, maternal effects of PI, and mechanisms of early MK, may all involve some form of genomic imprinting [Werren 2011; Negri and Pellecchia, 2012; Rabeling and Kronauer, 2013]. The currently strongest evidence for a role of epigenetics was found by Negri et al. [2009], who showed that *Wolbachia* interferes with host sexual differentiation in the leafhopper *Zyginidia pullula* by disrupting methylation patterns and genetic imprinting. In *Drosophila* species, *Wolbachia* prophage DNA adenine methyltransferase genes might be involved in the modification or rescue process of CI [Saridaki et al., 2011]. These studies are first indications for a role epigenetics in host manipulation, but we are only at the beginning of elucidating the precise molecular and biochemical pathways involved. Technological developments now allow for easier characterization of epigenetic marks, and transcriptome and proteome comparison of infected and uninfected individuals in various systems may be a promising way forward. Without doubt,

559 more mechanistic studies of host reproduction manipulation are going to reveal novel and
560 intriguing insights into the co-evolution between host and endosymbiont reproduction.
561
562
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Titles and legends to figures

Fig. 1. The four manipulation phenotypes of endosymbionts that affect different developmental stages of arthropods (using a butterfly life cycle as an example). Red arrows: thelytokous parthenogenesis induction (PI), purple arrows: cytoplasmic incompatability (CI), blue arrows: feminization (FM), green arrows: male-killing (MK), light green: early male killing (EMK) and dark green: late male killing (LMK) in terms of the developmental stage at which MK occurs. Each arrow indicates the corresponding host developmental stage at which endosymbiont manipulation takes place. The sex-determination-differentiation pathway is enlarged to depict the position in the gene cascade and timing during development at which endosymbionts interfere. *Transformer* (*tra*) is the central gear to transmit the primary signals to the conserved master switch gene *doublesex* (*dsx*), which regulates the downstream sexual differentiation. The question mark next to *tra* refers to insects in which *transformer* appears to be absent, such as Lepidoptera.

Fig. 2. Transitions between the four different manipulative phenotypes of *Wolbachia*. FM: feminization, EMK: early male killing, LMK: late male killing, CI: cytoplasmic incompatibility and PI: thelytokous parthenogenesis induction. The reported species (and orders) are indicated at each arrow, as well as their mode of sex determination.

Fig. 3. PI-inducing endosymbionts and haplodiploid host sex determination. CSD: complementary sex determination, MEGISD: maternal effect genomic imprinting sex determination. Red arrows: incompatible combinations. Green arrows: compatible combinations. CSD is only compatible with PI if diploidization is other than by gamete duplication (e.g. premeiotic doubling, central fusion or terminal fusion). MEGISD species can only have PI if the maternal imprint, that prevents female development, is not copied during gamete duplication, or the endosymbionts remove it before or after diploidization. The mode of sex determination and diploidization are mutually informative on each other: in *Lepidoptera clavipes* and *Trichagramma kaykai*, PI occurs by gamete duplication and CSD is excluded as sex

924 determination [Schilthuizen et al., 1998; Pannebakker et al., 2004; Tulgetske, 2010]; in
925 *Asobara japonica* CSD is absent and diploidization could be by gamete duplication [Kremer et
926 al., 2009; Ma et al., 2013]; and in *Encarsia hispida* feminization of diploid males can occur
927 under MEGISD if *Cardinium* removes the imprint [Giorgini et al. 2009].

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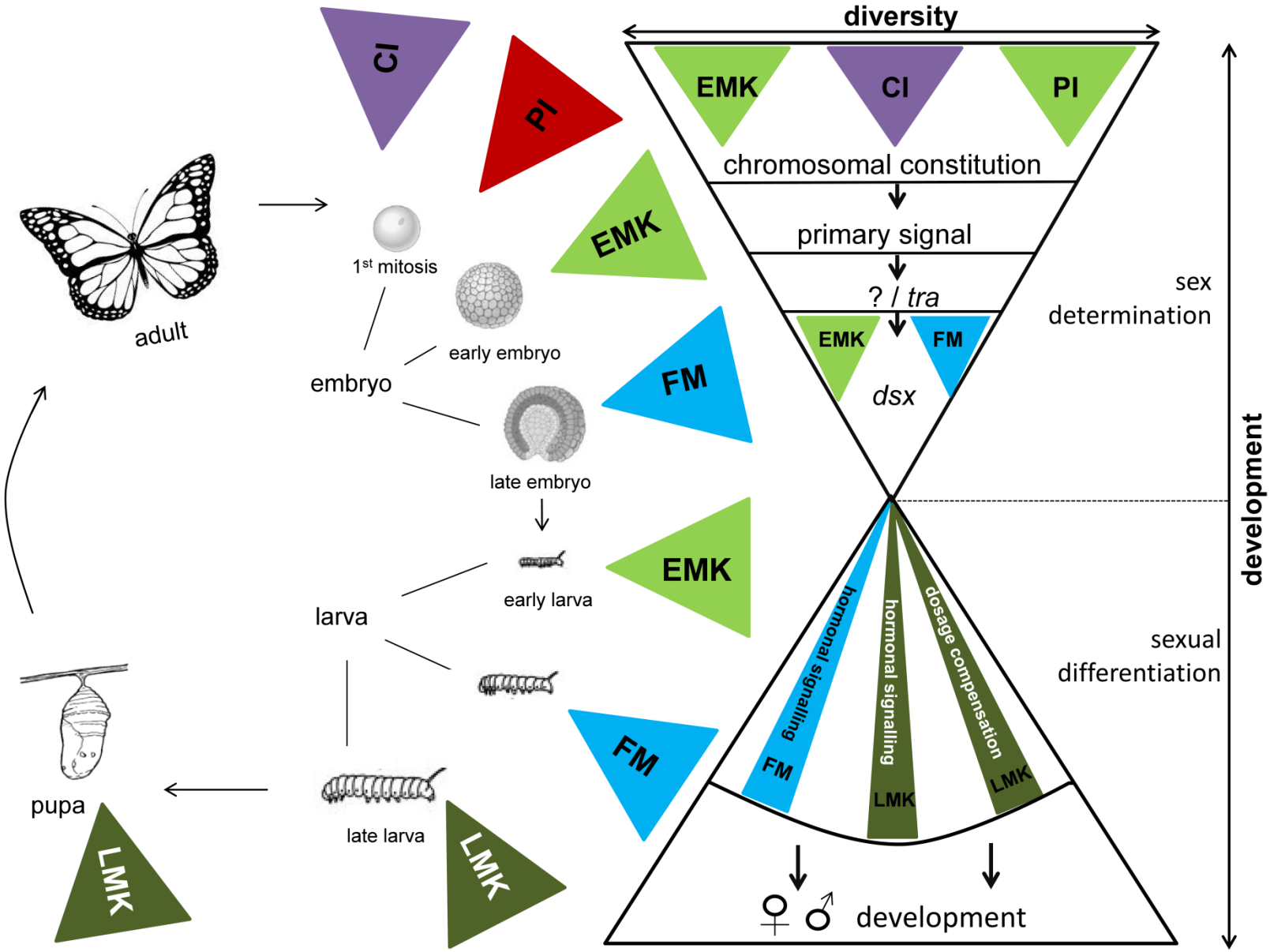
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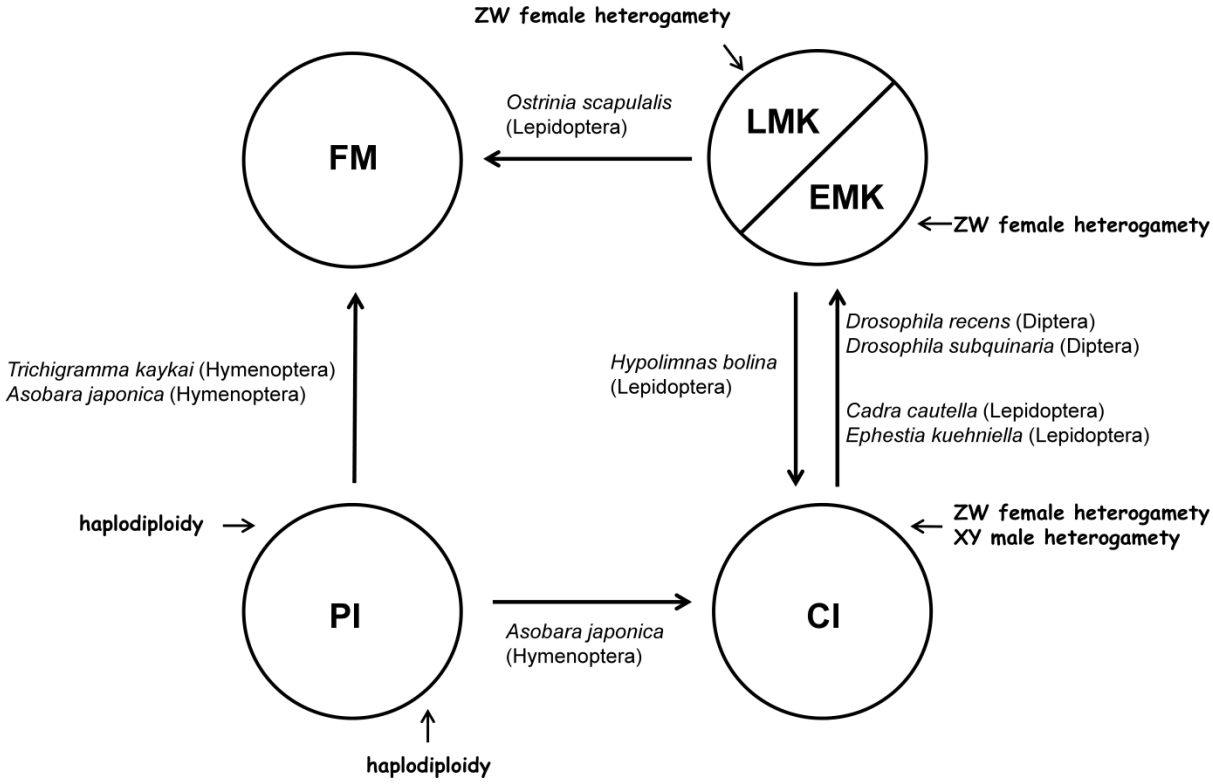
Fig. 1.



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931 **Fig. 2.**

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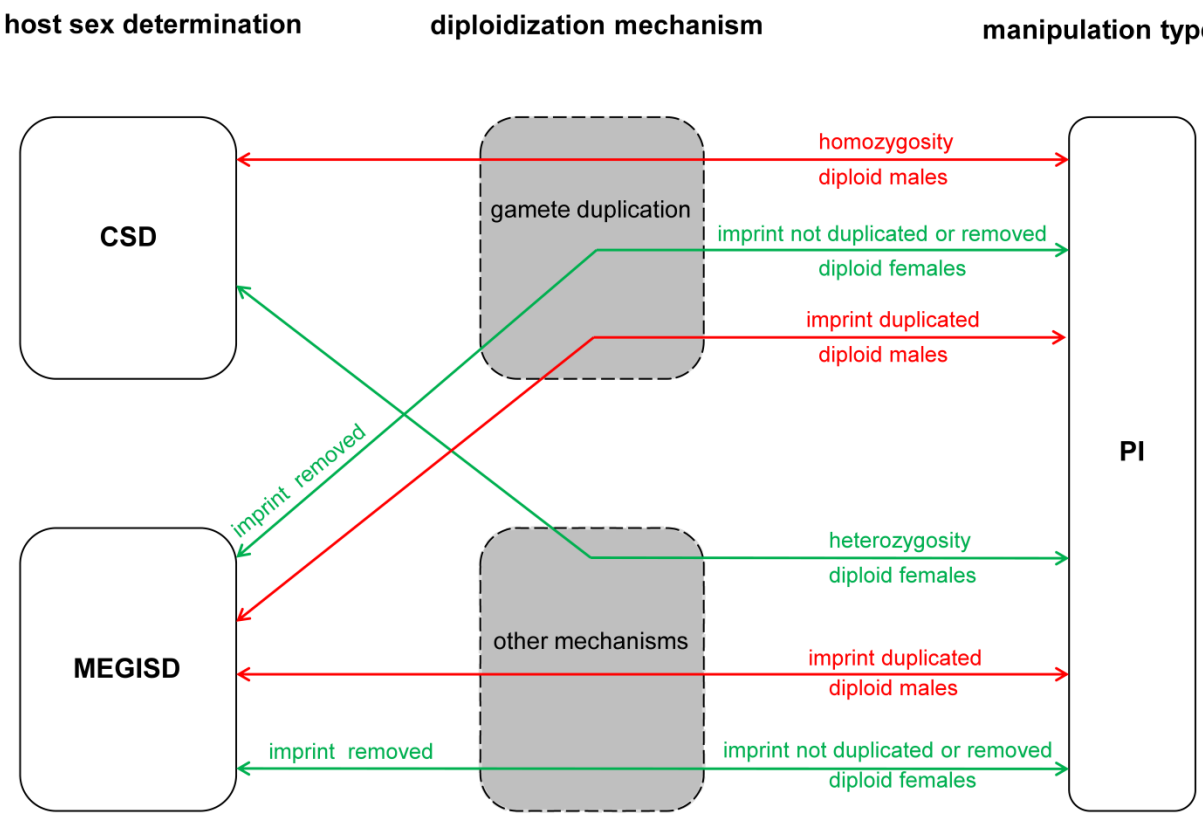

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940 **Fig. 3.**

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Table 1. Association between endosymbionts, arthropod host orders and host sex determination. Data are summarized from Kageyama et al. [2012].

Manipulation phenotype	Endosymbiont	Arthropod host order	Host sex determination (number of species reported)
Cytoplasmic incompatability (CI)	*Wolbachia*	Coleoptera	XY or XO male heterogamety (7)
	Cardinium	Diptera	XY or XO male heterogamety (18)
		Hymenoptera	haplodiploidy (9)
		Hemiptera	XY male heterogamety (3)
		Lepidoptera	ZW or ZO female heterogamety (5)
		Orthoptera	XO or XY male heterogamety (6)
		Isopoda	ZW female heterogamety (2)
		Trombidiformes	haplodiploidy (6)
		Mesostigmata	unknown (1)
Parthenogenesis (PI)	*Wolbachia*	Hymenoptera	haplodiploidy (24)
	Cardinium	Thysanoptera	haplodiploidy (1)
	Rickettsia	Trombidiformes	haplodiploidy (2)
Male killing (MK)	*Wolbachia*	Coleoptera	XY male heterogamety (4)
	Spiroplasma		ZW female heterogamety (4)
	Rickettsia		Unknown (3)
	Arsenophonus	Diptera	XY male heterogamety (14)
	Flavobacteria	Pseudoscorpiones	XO male heterogamety (1)
	Microsporidia	Hemiptera	XO male heterogamety (1)
	parasites	Lepidoptera	ZW or ZO female heterogamety (13)
	unknown virus	Hymenoptera	Haplodiploidy (1)
Feminization (FM)	*Wolbachia*	Lepidoptera	ZW or ZO female heterogamety (2)
	Cardinium	Hemiptera	XO male heterogamety (1)
	Microsporidia	Hymenoptera	haplodiploidy (2)
	parasites	Trombidiformes	haplodiploidy (3)
	Gasteromermis	Isopoda	ZW female heterogamety (2)
	f factor (unknown)		XY male heterogamety (1)
			unknown (2)
		Ephemeroptera	unknown (1)
		Amphipoda	unknown (4)

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